

**Interactomics: Protein Arrays and Label-Free Biosensors.**  
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**Lecture-3.**  
**An Overview of surface Plasmon Resonance (SPR).**

Welcome to MOOC interactomics course. In today's lecture we will talk about one of the very promising label-free technique, surface Plasmon resonance. It is an optical method based on surface plasmon resonance and evanescent waves which provides kinetic resolution of binding reactions in a label-free manner. The SPR spectroscopy bio-sensors are popular technology because of their simple instrumentation and high sensitivity, there also in great demand because they can provide label-free real time detection of various bio-molecular interactions. SPR is used in research for various application of biology including the drug discovery, clinical diagnostics as well as security applications.

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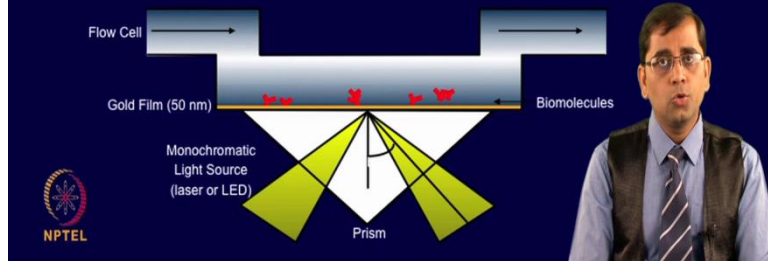
## Lecture Outline

- Principle of SPR
- Merits and demerits of SPR
- Some guidelines for SPR assay design and data analysis.



## Surface Plasmon Resonance (SPR)

- Measures change in refractive index of medium directly in contact with sensor surface (e.g. gold)
- Medium in contact with surface is commonly an aqueous sample containing analyte “protein”

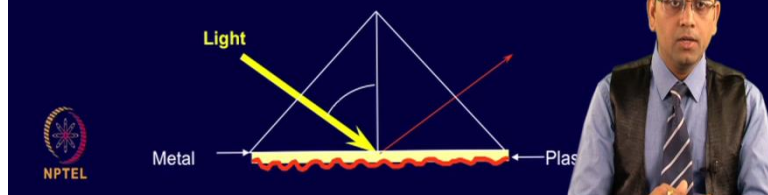


So, let us discuss, what is SPR? SPR is the surface sensitive spectroscopic method which measures the changes in the refractive index of the medium directly in contact with the sensor surface and the commonly employed metal is gold, as shown in the slide that there is a prism, a light source, antibody immobilized on the gold surface and flow cell from which we can inject the target proteins to be studied. So, medium in contact to the surface is commonly an aqueous sample containing the analyte protein.

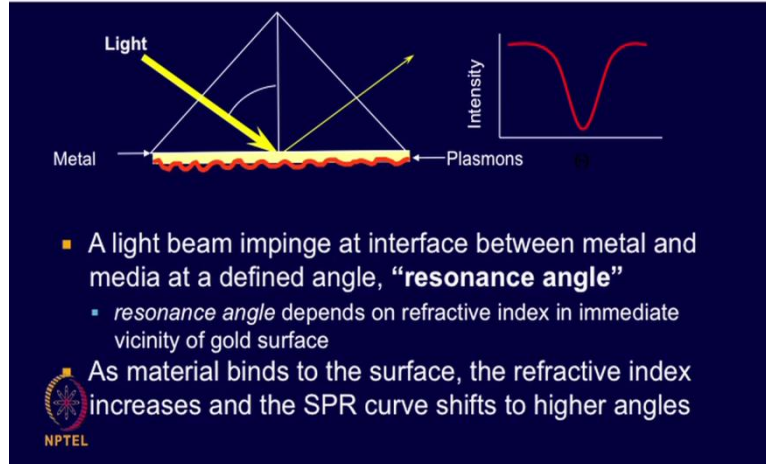
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## SPR (contd.)

- Plasmons - special electromagnetic waves that can be excited at certain metal interfaces, mostly gold & silver
  - generated on boundary of metal & external medium (e.g. air)
  - very sensitive to any change of this boundary (e.g. adsorption of biomolecules to the metal)



## SPR (contd.)




Surface Plasmons are special electromagnetic waves that can be excited at certain metal interfaces; mostly gold and silver are used for this purpose. The surface plasmons are electromagnetic waves that propagate parallel to the metal or dielectric surface from that interface the plasmons are created in the light energy from polarized incident photon is coupled in the oscillation mode of free electron density which is present in the metal field. From these gold surfaces these Plasmons are generated at the boundary of metal and external medium which is usually air.

These are very sensitive to any changes on this boundary like adsorption of bio-molecules to the metal. In SPR, a light beam impinges at the interface between metal and media at a defined angle called as resonance angle. The resonance angle depends upon the refractive index in immediate vicinity of the gold surface. When metal binds to the gold surface the refractive index increases and the SPR curve shift toward the higher angles. So, these changes in the angle of refraction of light caused due to the binding of probe to the immobilized proteins are measured for the characterization of bio-molecular interactions in real time.

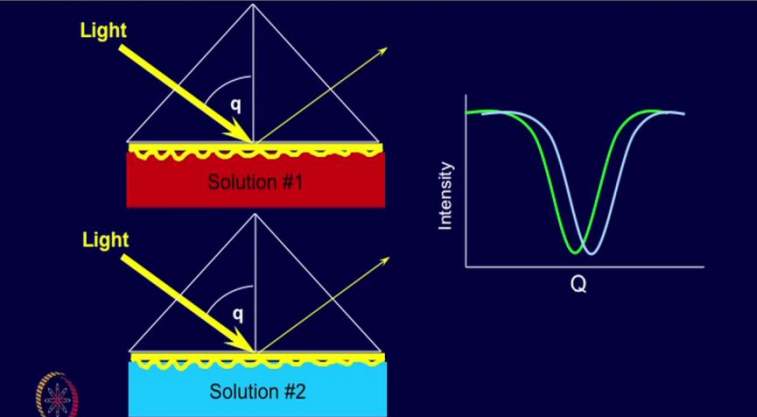
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## Real-time label-free detection by SPR

- Excitation of a planar surface with light excites surface plasmons and changes reflectivity
- Real-time label-free detection of binding events detected by measuring change in SPR reflectivity
- Changes in refractive index are monitored continuously
- Platform for real-time label-free detection



**SPR angle**



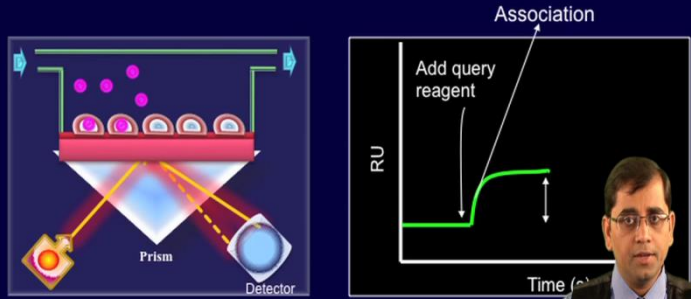
SPR angle depends on refractive index near the surface

The diagram illustrates the SPR setup. It shows two identical prism-solution systems. The top system is labeled 'Solution #1' and the bottom 'Solution #2'. In both, a yellow light beam is incident on a gold surface at an angle  $q$ . The reflected light is shown as a dashed line. To the right, a graph plots 'Intensity' on the y-axis against 'Q' on the x-axis. Two curves are shown: a blue curve with a deeper minimum and a green curve with a shallower minimum, both centered at the same Q value.



Now, in this slide let us look at the SPR angle., how it depends on refractive index near the surface and the SPR angle which is directly related to the amount of bio-molecules binding on the gold surface. The real time label-free detection of binding events can be detected by measuring changes in SPR reflectivity. These changes in refractive index are continuously monitored to obtain the kinetic data in real time manner making it a remarkable label-free detection technique.

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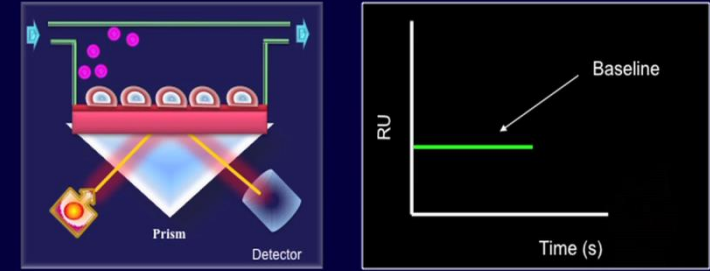
### SPR Sensorgram (3)




The diagram on the left shows a flow cell containing a gold-coated chip with immobilized ligands. A light source and a detector are positioned to measure the SPR signal. The graph on the right plots Resonance Units (RU) against Time (s). A horizontal line represents the baseline. An arrow labeled 'Add query reagent' points to the start of a step increase in the RU signal, which is labeled 'Association'.



### SPR Sensorgram (2)



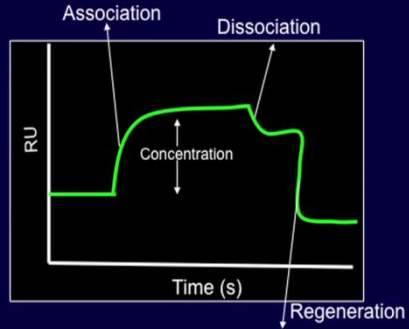
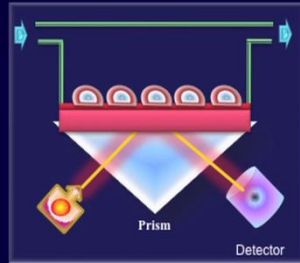
The diagram on the left is identical to the one in slide (3). The graph on the right plots Resonance Units (RU) against Time (s) and shows a single horizontal line labeled 'Baseline'.



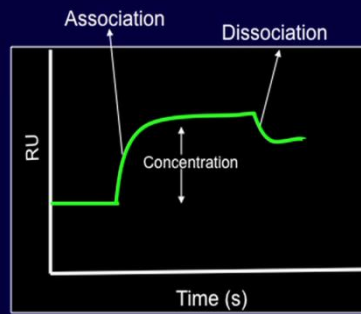
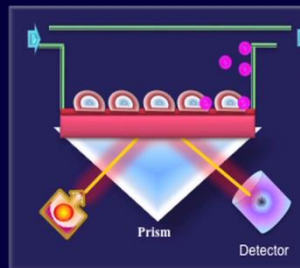
Let us now discuss SPR sensor grams. The sensor grams describe the changes in SPR signal verses time as molecules bind and dissociate from the sensor surface, the resulting change in resonance signal creates a sensor gram. Let us look at the various steps involved in these sensor grams. As you can see this slide, there is prism, light source, a gold coated chip and ligand immobilized on the chip surface, initially the running buffer is injected on to the immobilized surface, it generates a base line. The baseline dummy is straight until the query molecule or the analyte is injected in the medium.

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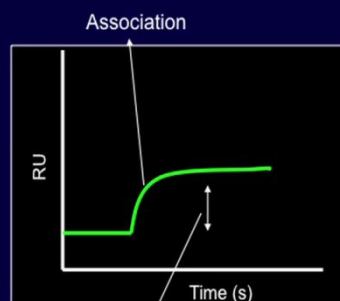
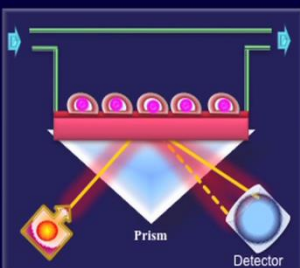
## SPR Sensorgram (6)



## SPR Sensorgram (5)



## SPR Sensorgram (4)



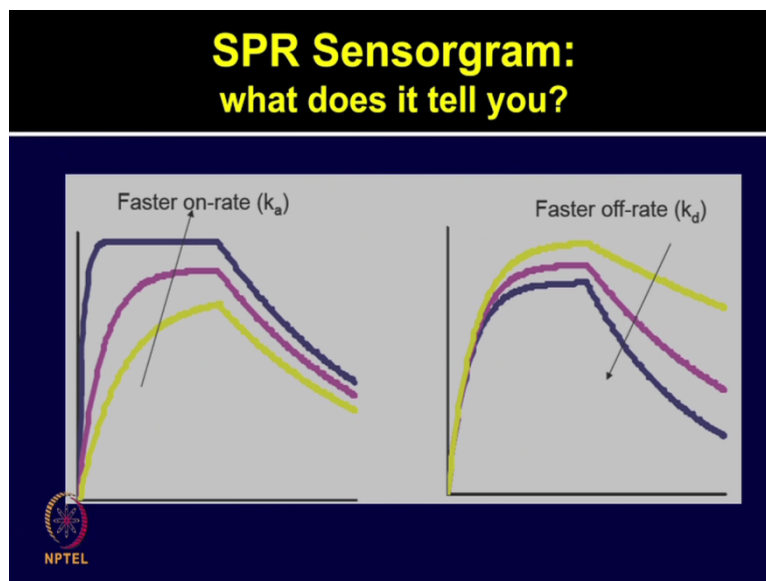
Stochastic steady state signal =  
RU (after)-(before)



So, initially the surface is washed with the running buffer followed by injection of the query molecule in the same running buffer, as the analyte starts interacting with the ligand association phase can be observed in SPR sensor gram from which association rate  $k_{on}$  or  $k_a$  can be derived. In this slide, you can see some of the query molecules have started to bind the ligand immobilized on the chip surface, note, sometime when the association achieves a saturation level, they reach to a state known as stochastic steady state.

Injection of running buffer at this point helps in analyte dissociation from which the dissociation rate  $k_{off}$  or  $k_d$  can be derived. As shown here in the right panel initially, you generate a base line followed by an association phase and then a dissociation phase. The left panel is also showing proteins being dissociated from bound molecules. So, after the run is completed, the same chip can be reused for further runs but one needs to perform mild acid or base treatment on the surface and further washing with the running buffer. This process of complete removal of analyte from the chip surface is known as regeneration.

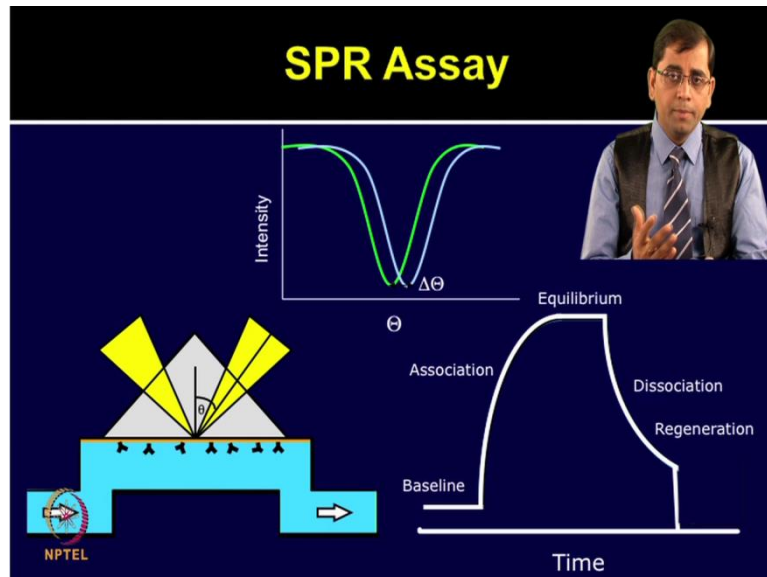
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So, what this sensor gram tells us, these curves, shapes and amplitude of binding measurement can be used to determine the kinetics of interaction and concentration analysis. The analysis of SPR sensor gram can answer many questions regarding the specificity, the affinity, kinetics, concentration and thermodynamics. So, the shape and amplitude provides various information by looking at these SPR sensor gram curves. As shown here in this slide, the on rate and off rate can provide this information as to whether it is slow or fast. In addition comparison of various analytes can be made simultaneously for their association and

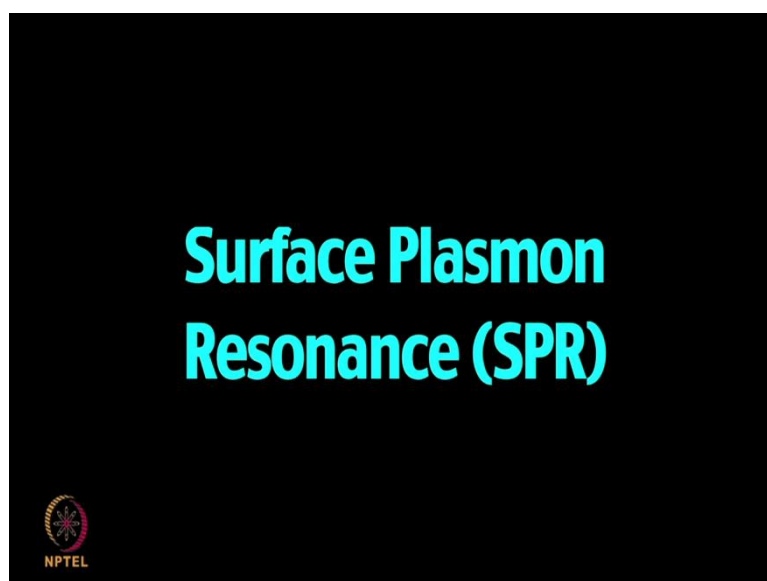
dissociation rates by looking at these SPR sensor grams. So, overall the SPR assay means tracking the SPR angle to measure the binding events.

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Now, by looking at some basic concepts and studying the details of SPR sensor grams. I think, it should be cleared as to how SPR angle is used to measure these binding interactions. As shown here, initially you observe a baseline after binding even happens association phase can be seen following which it may reach a steady state. This followed by buffer wash shown as dissociation, the same surface can be reused after regeneration for further experiments.

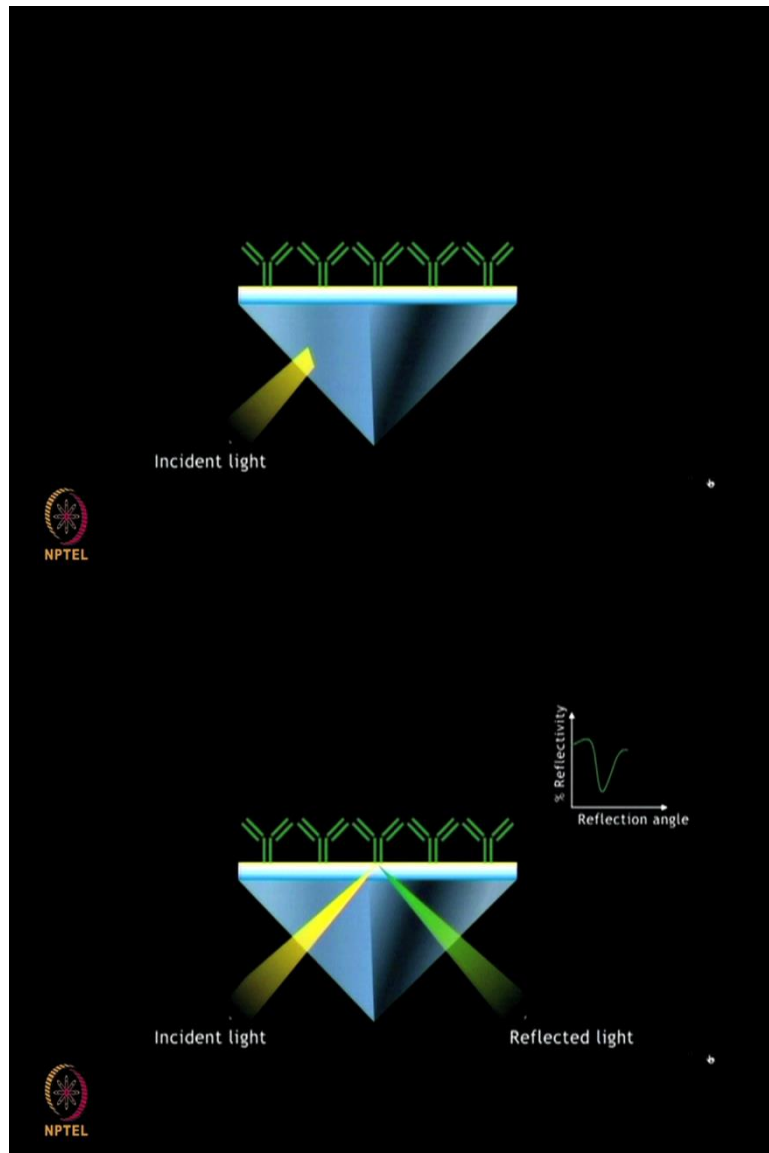
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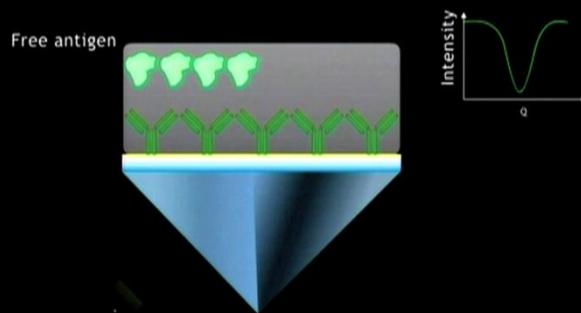
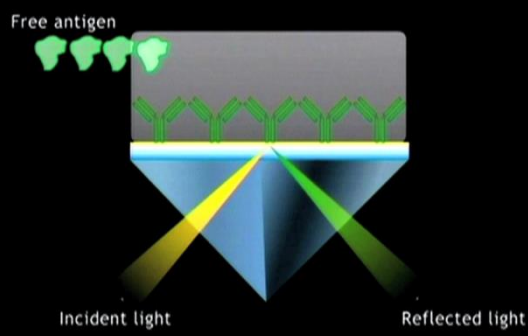
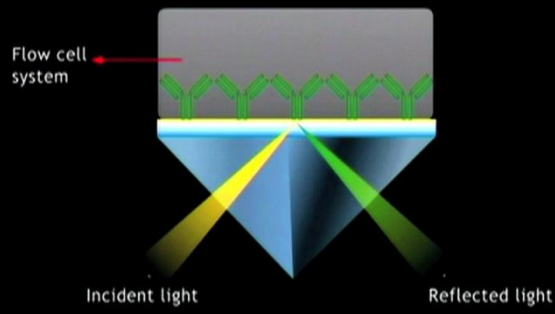


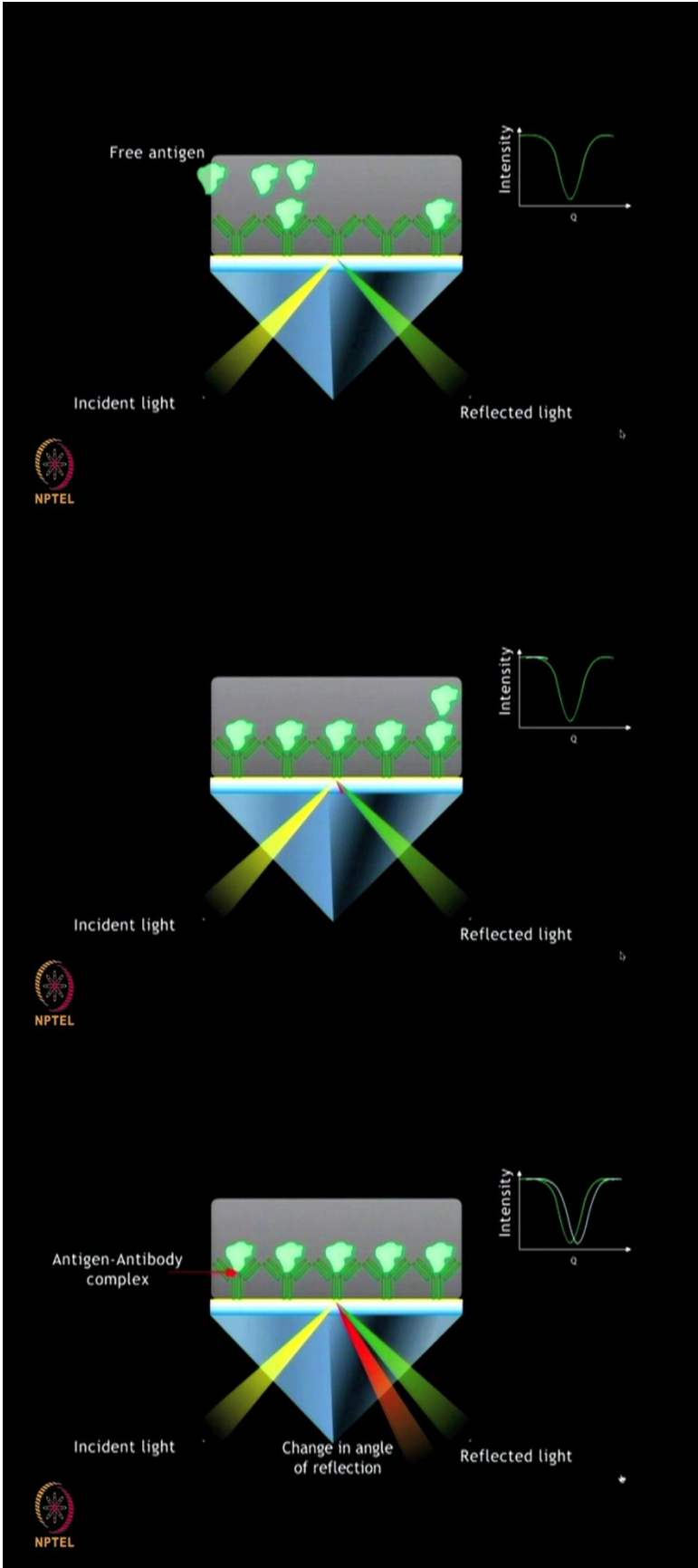


So, now, we will view an SPR animation to understand some other the basic concepts. Surface Plasmon resonance SPR is a highly sensitive, a spectroscopic tool that is increasingly being used for label-free detection studies. Test proteins such as antibody are immobilized on to the gold coated glass array surface incident light is striking the surface is constantly reflected at a particular angle in this state

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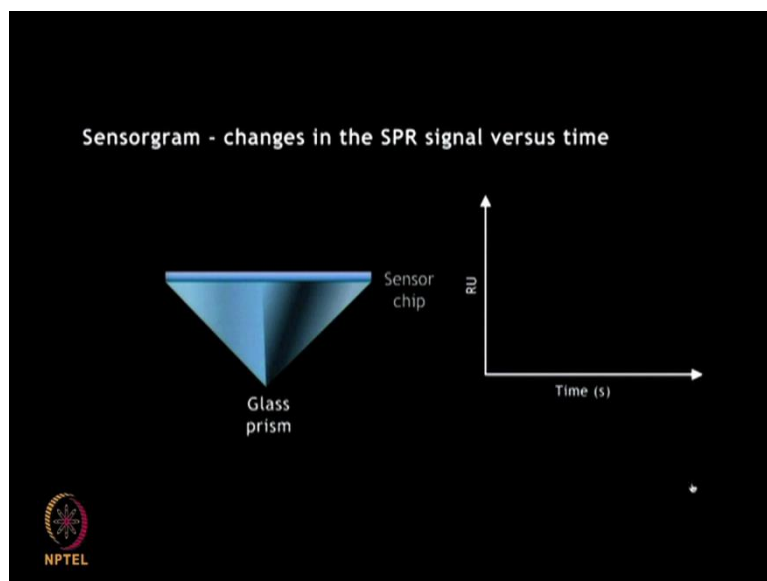


So, let us watch this animation where gold film on top of glass slide then there is a prism, the dust antibodies are immobilized on the gold surface. The incident light strike the surface is constantly reflected at a particular angle. In SPR experiments, the unlabeled free antigens or other query proteins enter via the flow cell and move towards the immobilized antibody or other test proteins.

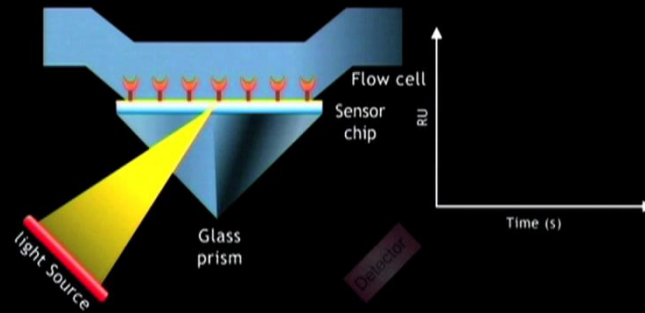
Initially, there is no change in reflected light; the binding of antigen to a antibody immediately brings about a change in the angle of reflection of light due to changes in the refractive index of the medium. These changes can be continuously monitored to characterized bio-molecular interactions in real time.

The SPR angle or the angle at which minimum intensity of reflected light is obtained is indicative of the amount of biomolecule binding to the surface. The graph shown on the right side represents change in reflection intensity before and after the antigen binding.

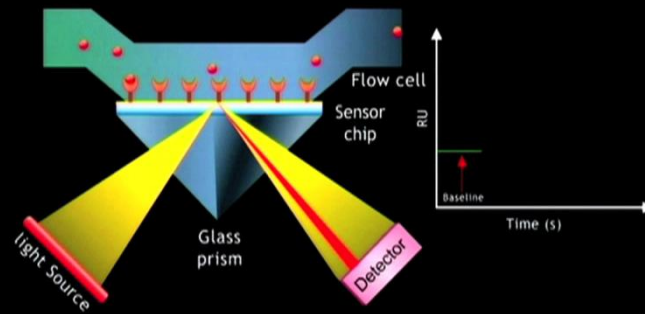
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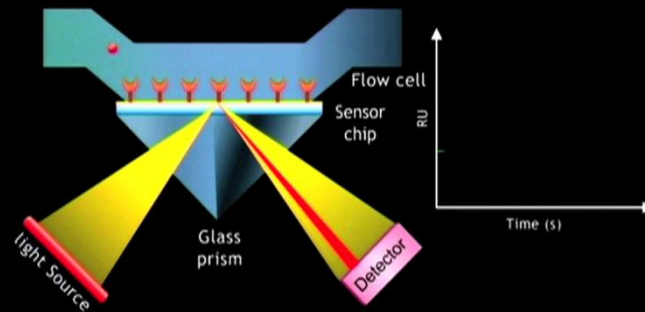
Sensorgram - changes in the SPR signal versus time



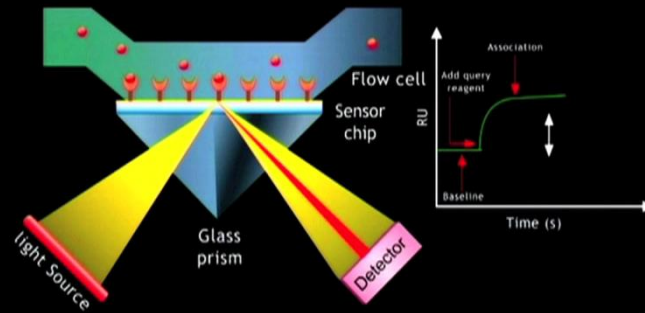
Sensorgram - changes in the SPR signal versus time



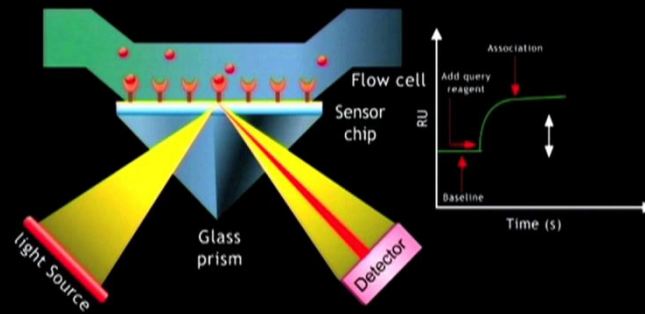
Sensorgram - changes in the SPR signal versus time



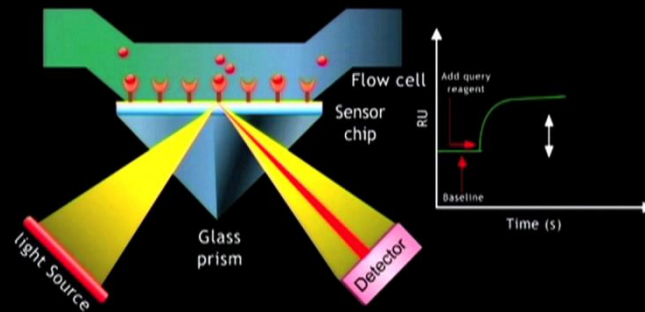
Sensorgram - changes in the SPR signal versus time



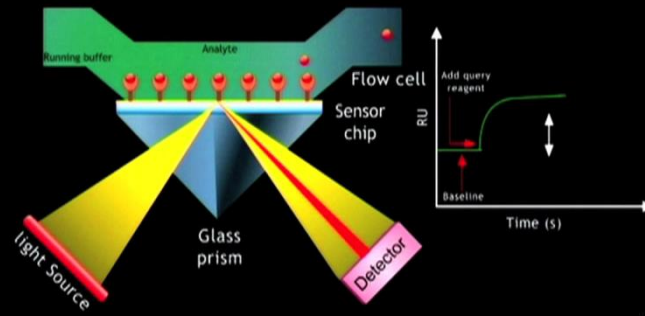
Sensorgram - changes in the SPR signal versus time



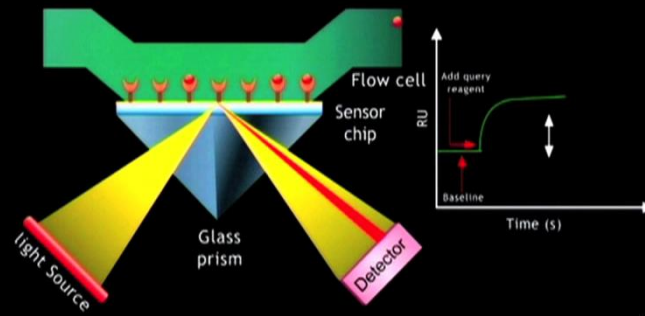
Sensorgram - changes in the SPR signal versus time



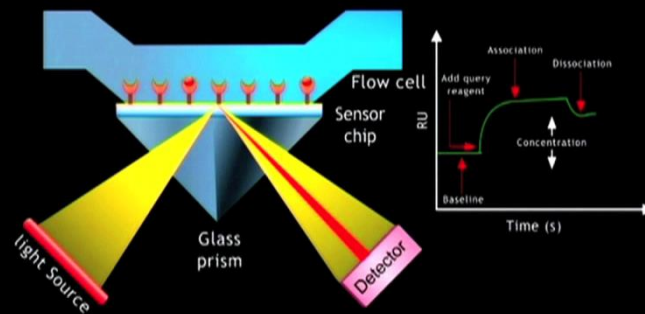
### Sensorgram - changes in the SPR signal versus time

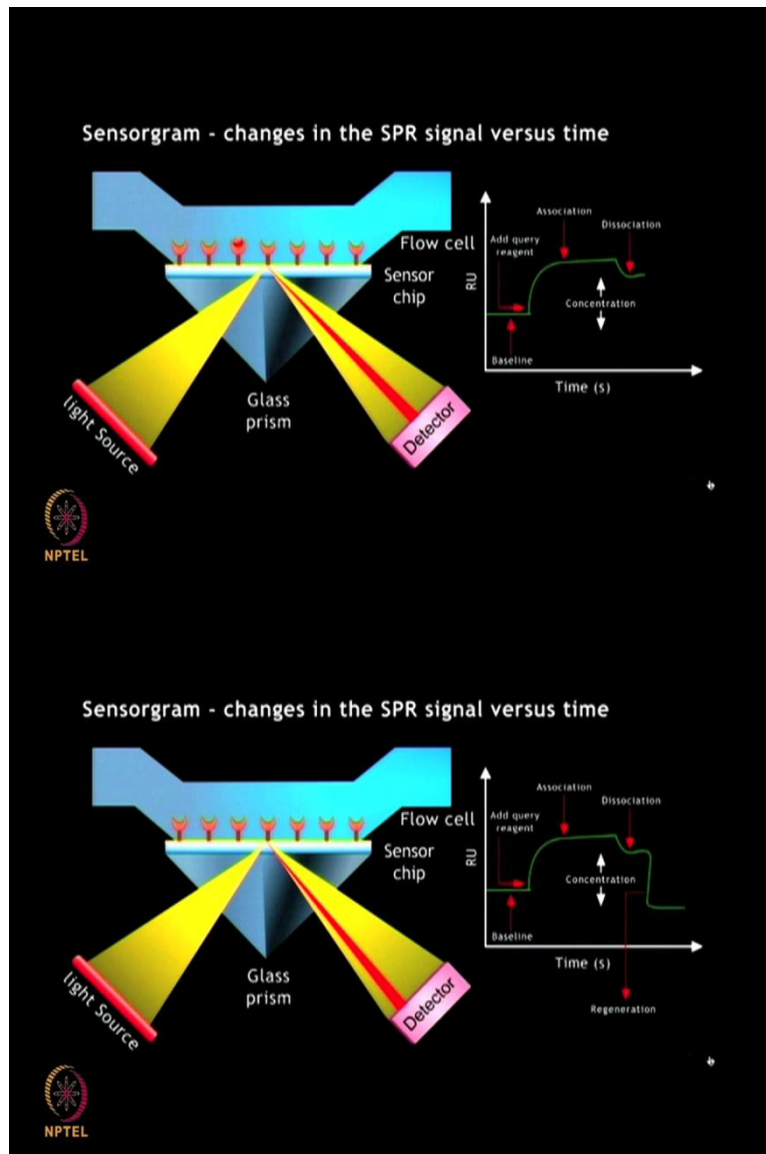


### Sensorgram - changes in the SPR signal versus time



### Sensorgram - changes in the SPR signal versus time





SPR sensor grams, the sensor gram describes changes in SPR signal versus time. Initially, the running buffer is added on to SPR chip. So, in animation the prism, light source, gold slide, immobilized antibodies are shown. So, when the running buffer is added on to the SPR chip containing printed antibodies initially, the baseline is straight. When query molecule is added in flow cell, the interacting antigen binds to the antibody and association can be seen in SPR sensor gram.

Now, the graph is showing association or on rate  $K_a$ , after sometime the binding reaches to a saturation level known as stochastic steady state. When running buffer is further added, the bound proteins are dissociated which can be seen as a dissociation rates in the graph which is represented by off rate or  $K_{off}$ . After the SPR run is finished, same chip can be reused by applying a mild acid treatment and further washing steps by a process known as regeneration.



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## SPR advantages

- Label-free
  - No need for tedious or expensive labeling protocols
  - Avoid potential labeling artifacts
- Direct
  - Measures binding of the actual analyte
- Measure binding kinetics and affinity ( $k_a$ ,  $k_d$ , KD)
- Real-time
  - Allows user to watch the experiment as it happens
  - Not an “end point assay”




There are many advantages of using SPR; first of all, it is a label-free method. So, there is no need of addition of tags by following tedious labeling methods. It avoids (15:17) due to the labeling. It is a direct method because it provides measurement of binding of actual analyte. It provides information in real time as the experiment proceeds. Therefore, it is not an end point assay unlike other label-based detection methods and most importantly, it gives you measure of binding kinetics and affinity, the on rate, off rate and dissociation constant; however SPR also has certain limitation.

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## SPR limitations

- SPR detection relies on mass changes
- SPR detection decreases exponentially with distance from surface
- Estimated detection limit ~200 nm
- Limited to choice of metal which results in SPR



## SPR limitations (2)

- Sample must be homogeneous
- Sample preparation and probe attachment to metal surface can be difficult
- Non-specific interactions also results into SPR signal
  - Need to ensure specific signal
  - Avoid bulk effects
- Refractive index is temperature dependent




SPR detection relies on mass changes and it decreases exponentially with distance from the surface. It is estimated that approximate detection limit is around 200 nanometer. Additionally, it is also limited to the choice of metal such as gold and silver which can result in surface plasmons. Regarding the samples you cannot use very viscous or every kind of samples for SPR analysis Sample need to be homogeneous and the sample preparation has to be very meticulous.

The immobilization procedure may require lot of optimization with different type of surface chemistries, often non-specific interaction can also result in fast SPR signal. So, there is need to ensure that the signals obtained are specific and unique to the experiments, the bulk effect can also interfere with the actual data which needs to be avoided. Lastly, refractive index is also temperature dependent. So, these are some of the limitations associated with SPR however, it is a still one of the very promising label-free detection platform.

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## Points to Ponder

- SPR is the surface sensitive spectroscopic method which measures the changes in refractive index of the medium directly in contact with the sensor surface.
- As molecules bind and dissociate from the sensor surface, the resulting change in resonance signal creates a sensorgram.
- Sensorgrams describe the changes in SPR signal versus time.
-  The process of complete removal of analyte from the chip surface is known as regeneration.

## Points to Ponder (cont.)

- SPR provides data in real time without the need of addition tags and avoiding artifacts due to the labelling.
- The assay is limited to the choice of metal which results in surface plasmons such as gold and silver.

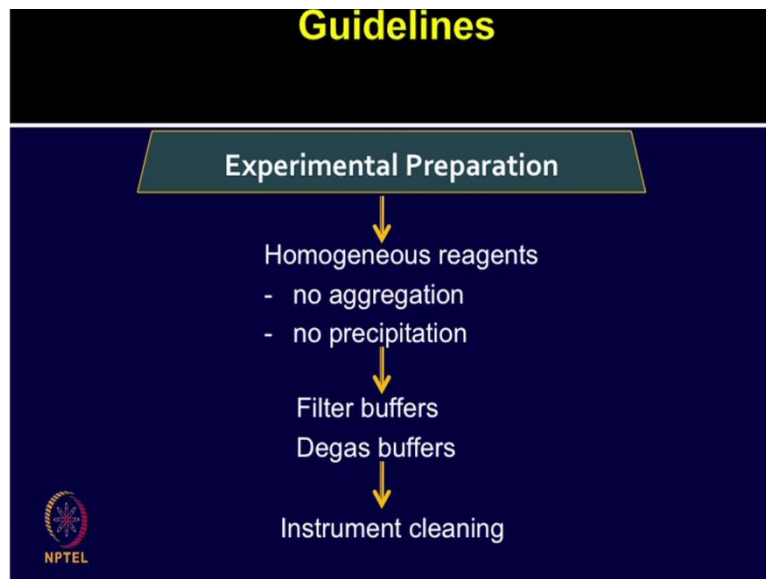


Guidelines for assay design and  
data analysis



Let us now discuss the guidelines for performing SPR experiments and its data analysis. Performing a good SPR experiment and accurate interpretation of binding reactions from these biosensor is always very challenging. David Visca from university of Utah in United States has provided very detailed guideline for biosensor analysis. I will briefly describe some of these guidelines which can be used for performing SPR experiments and data analysis.

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First of all, experimental preparation. It is very important to start with quality reagents that ensure high quality data. Reagents should be homogeneous; there should not be any aggregates or precipitation. Filtration and degassing of buffers is important because even a small bubble can ruin the whole experiment. Additionally, instrumental cleaning is very important, any dust particle or any type of contaminant can result in artifacts.

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## Guidelines (2)

### Surface Capacity



Controls

- Empty surface
- Single component
- Baseline check



## Guidelines (3)

### Experimental Parameters



Injection at a fast flow rate ( 50-100  $\mu\text{L}/\text{min}$ )



Analyze blanks of running buffer periodically



Replicates: slides and/or sample



## Guidelines (4)

```
graph TD; A[Data Processing] --> B[Double reference]; B --> C[Subtract response from reference surface]; C --> D[Subtract response of buffer injection];
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## Guidelines (5)

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graph TD; A[Data Analysis] --> B[Global fit data set]; B --> C[Use appropriate fitting models];
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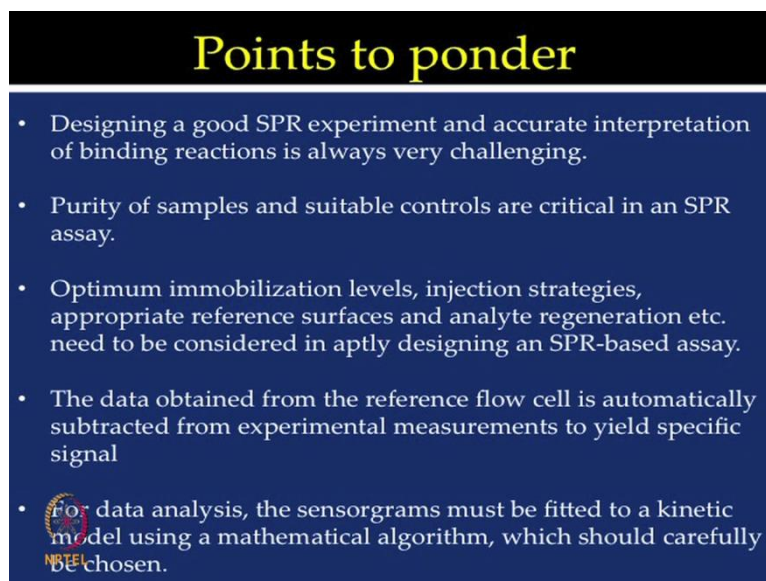
Ideally, the analyte and the ligand should be monomeric in solution able to form one to one complex for the data to fit into the simple reaction model. Surface capacity is another important consideration. What kind of controls need to be used? What reference empty surface needs to be used? Single component binding and baseline checks, all of these are important points to consider.

Low sensor surface capacity is preferable in many cases which can help in minimizing issues such as mass transport, aggregation and steric hindrance. There are various experimental parameters which one needs to consider, for example, how fast or how slow the injection rate should be? Ideally, the fast flow rate can minimize mass transport type of effects, analyzing blanks of running buffer periodically is critical to ensure reliable results and stable baseline.

Also, it is always tedious to reproduce such experiments on independent slides and independent samples. Data processing, one of a need to do double referencing specially if we are talking about SPR data, subtract the response from the reference surface and subtract response of buffer injection. Subtracting the reference surface data from the reaction surface can reduce the issues which are related to refractive index changes. The double referencing is the blank injection response which is reduced to remove artifacts.

Now, how to fit the data to selection of appropriate fitting models is always very critical. The experiment must be designed accurately. As an example, for an antigen and antibody interaction, for an antibody being able to bind to two different antigens, it should be used as a ligand and antigen should be flown over as analytes to study their biomolecular interactions. If binding data has to be correlated to interaction modules, experiments should be designed critically to be able to fit to the data by using appropriate fitting models.

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### Points to ponder

- Designing a good SPR experiment and accurate interpretation of binding reactions is always very challenging.
- Purity of samples and suitable controls are critical in an SPR assay.
- Optimum immobilization levels, injection strategies, appropriate reference surfaces and analyte regeneration etc. need to be considered in aptly designing an SPR-based assay.
- The data obtained from the reference flow cell is automatically subtracted from experimental measurements to yield specific signal
- For data analysis, the sensorgrams must be fitted to a kinetic model using a mathematical algorithm, which should carefully be chosen.

In summary, Today we talked about surface Plasmon resonance which monitors biomolecular interactions in label-free manner moreover, it provides quantitative information for the binding kinetics. We also discussed about its strengths and weakness of the approach and guidelines to follow the SPR assays; we will continue our discussion on SPR imaging in next lecture. Thank you.

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## Summary

- SPR is a surface sensitive optical method which measures the changes in refractive index of the medium directly in contact with the sensor surface.
- The method allows monitoring of many label-free molecular interactions in parallel to provide information on kinetic rates and binding affinities.
- Sensorgrams describe the changes in SPR signal versus time.

• Experimental design of SPR assay, data processing and analysis are critical to obtain reliable results.



## References

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